Binding of phytochrome B to its nuclear signalling partner PIF3 is reversibly induced by light

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The phytochrome photoreceptor family directs plant gene expression by switching between biologically inactive and active conformers in response to the sequential absorption of red and far-red photons[1,2]. Several intermediates that act late in the phytochrome signalling pathway have been identified, but fewer have been identified that act early in the pathway[3,4]. We have cloned a nuclear basic helix–loop–helix protein, PIF3, which can bind to non-photoactive carboxy-terminal fragments of phytochromes A and B and functions in phytochrome signalling *in vivo*[5]. Here we show that full-length photoactive phytochrome B binds PIF3 *in vitro* only upon light-induced conversion to its active form, and that photoconversion back to its inactive form causes dissociation from PIF3. We conclude that photosensory signalling by phytochrome B involves light-induced, conformer-specific recognition of the putative transcriptional regulator PIF3, providing a potential mechanism for direct photoregulation of gene expression.

The phytochromes are chromoproteins encoded by a gene family of five members, *PHYA* to *PHYE*, in *Arabidopsis*[6]. Each photoreceptor molecule consists of a dimer of two ~125K subunits with a single covalently linked tetrapyrrole chromophore. The 125K polypeptide folds into two major structural domains, each encompassing about half of the molecule: an amino-terminal domain that cradles the chromophore, and a carboxy-terminal domain that mediates dimerization[7]. Changes in gene expression are triggered by photoconversion of the photoreceptor to its active (Pfr) form[8,9]. Several components have been cloned that are involved in signal transduction beyond the point of convergence of the phytochrome and blue/ultraviolet-light-receptor pathways[4,10,11]. PIF3, a putative transcription factor and phytochrome signalling partner, was isolated by virtue of its interaction with the non-photoactive C-terminal domains of phytochromes A and B (ref. 5). We now investigate whether this binding reaction is photoregulated in the full-length photoactive phytochrome B molecule.

We synthesized radioactively labelled full-length *Arabidopsis* phytochrome B by *in vitro* transcription–translation, ligated the chromophore to the protein, and measured the binding of the holoprotein to matrix-immobilized PIF3 in response to pulse irradiations. Figure 1 shows that a 5-min pulse of red, but not far-red, light induces binding of phytochrome B to PIF3. Five minutes of far-red irradiation immediately after an initial red pulse reversed the red-light effect, so that no binding of phytochrome B to PIF3 was detectable. These results indicate that the binding of full-length phytochrome B to PIF3 is photoregulated, occurring predominantly, if not exclusively, in the biologically active Pfr form.

As this experiment involved a two-hour incubation in the dark after pulse irradiation, it was not clear whether giving a far-red pulse after a red pulse prevented phytochrome B from binding or caused it to dissociate after binding. We therefore followed the time course of red-pulse-induced binding and the response of bound phytochrome B to a delayed far-red pulse. Figure 2 shows that binding was initially rapid during the dark incubation period at 4 °C after the red-light pulse, then increased more slowly up to 4 h. A far-red pulse given 2 h after the initial red pulse caused a rapid decrease in bound phytochrome B to background levels (Fig. 2). These results show that phytochrome B can bind to PIF3 in darkness after pulse photoconversion to its active form, without the need for continuous photoactivation, and that once bound, reconversion to its inactive Pr form causes the rapid dissociation of phytochrome from PIF3.

We have shown previously that a set of missense mutations clustered in the C-terminal domains of phytochromes A and B

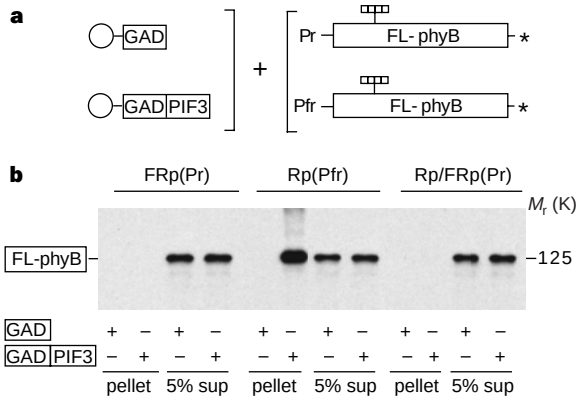

Figure 1 Phytochrome B (phyB) binds to PIF3 specifically as the biologically active conformer Pfr. **a**, Experimental design. Recombinant Gal4-activation domain (GAD) or GAD–PIF3 fusion protein, immobilized on beads (represented by circles) were each mixed with radioactively labelled (asterisks), chromophore-ligated (represented as small striped bars), full-length (FL) phyB, which was converted to either the inactive (Pr) or Pfr form by 5-min pulses of far-red or red irradiation, respectively. **b**, Autoradiography, of pelleted phyB and 5% of supernatant (5% sup) obtained by centrifugation after 2 h incubation in the dark at 4 °C, in the presence (+) or absence (–) of either GAD or GAD–PIF3, after pulse irradiation with 5 min far-red light (FRp), 5 min red light (Rp), or with 5 min red followed immediately by 5 min of far-red light (Rp/FRp).

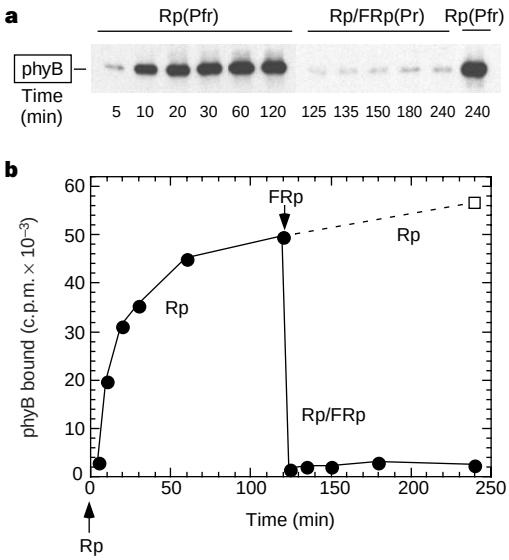

Figure 2 Induced binding of the active (Pfr) form of phytochrome B to PIF3 is reversed by photoconversion back to the inactive Pr form. **a**, Autoradiograph showing the time course of full-length phyB association with matrix-immobilized GAD–PIF3. Samples were irradiated with a 5-min pulse of red light (Rp), incubated in darkness at 4 °C for the periods indicated up to 120 minutes, and then either incubated further to 240 min or given a pulse of 5 min of far-red light (Rp/FRp) and incubated for the periods indicated up to 240 min before centrifugation. **b**, Quantitative determination of the amounts of PIF3-bound, radioactively labelled phyB shown in **a**.

interferes with the transfer of signals from the photoreceptor to transduction pathway components *in vivo*, without affecting photosignal perception[2,12,13]. Figure 3 shows that the three missense mutations tested here impair binding of photoactivated phytochrome to PIF3. These results are consistent with the conclusion that the reduced signalling by the mutant proteins *in vivo* is caused by a weakened interaction with PIF3, perhaps as a result of a reduction in binding affinity.

We investigated the relative functional importance of the N- and C-terminal domains in the interaction with PIF3 by comparing the binding to PIF3 of full-length phytochrome B with that of each separate domain, as well as with the apoprotein. Figure 4 shows that PIF3 binds strongly to photoactivated full-length phytochrome B but only moderately to the phytochrome B N-terminal domain and comparatively weakly to the C-terminal domain. These results indicate that there are binding determinants in each half of the protein that are strongly synergistic in the full-length molecule. This synergism requires the chromophore and conversion to the active conformer, because it is not seen in the full-length apoprotein or in the inactive form of phytochrome B, indicating that photoconversion to the active form drives the creation of a high-affinity binding site(s) from elements of each domain of the photoreceptor. It is unclear whether these elements are two or more separate sequences in the polypeptide or the residual 'halves' of a disrupted single site that would normally span the junction between the two domains. The red-light-induced binding of the isolated N-terminal domain to PIF3 indicates that the binding element in this domain undergoes a conformational change upon photoactivation. It is not known whether the C-terminal element can also undergo such a photoinduced switch in conformation when coupled to the chromophore-bearing domain in the full-length photoreceptor, or whether it remains constitutive.

The interaction of PIF3 with the C-terminal domain of phytochrome B, which appeared to be substantial in a previous pull-down experiment[5] seems to be relatively weak in our present assay. Preliminary evidence indicates that the lower concentrations of phytochrome B used in the binding reactions described here may explain this difference (data not shown).

Our results support the conclusion that PIF3 is a primary signalling partner of phytochrome B. At least three steps in the transduction process from phytochrome B to PIF3 could be reversibly dependent on the light-induced formation of the biologically active conformer of phytochrome B: the translocation of phytochrome B from the cytoplasm to the nucleus; binding of phytochrome B to PIF3; and the biochemical transaction constituting signal transfer to PIF3. Phytochrome B is induced to translocate from the cytoplasm to the nucleus when *Arabidopsis* seedlings are exposed to red light[14]. This would regulate access to PIF3, which may be constitutively nuclear[5], and indicates that photoactivated phytochrome B interacts specifically with nuclear import components. Our results show that phytochrome B is rapidly induced to associate with, and subsequently dissociate from, PIF3 upon sequential photoconversion to its active and inactive forms, respectively. The rapid reversible binding of the two partners caused by this photoinduced switching between the active and inactive states of the photoreceptor could be critical for regulating signal flow to PIF3. There is evidence for autophosphorylation of phytochrome A, suggesting that the phytochromes may be photoregulated serine/threonine kinases[15]. We do not know whether signal transfer from phytochrome B to PIF3 involves a photoregulated biochemical reaction such as *trans*-phosphyrolation, or an allosteric effect on some activity such as DNA binding. Nevertheless, several steps in the primary phytochrome signal transduction process are apparently subject to photoregulation.

Our results verify that the phytochrome molecule functions as a unique binary optical storage device, whose biochemical output is controlled by the stored information. Because PIF3 is a putative transcriptional regulator, it seems likely that this biochemical output directly regulates target gene expression. □

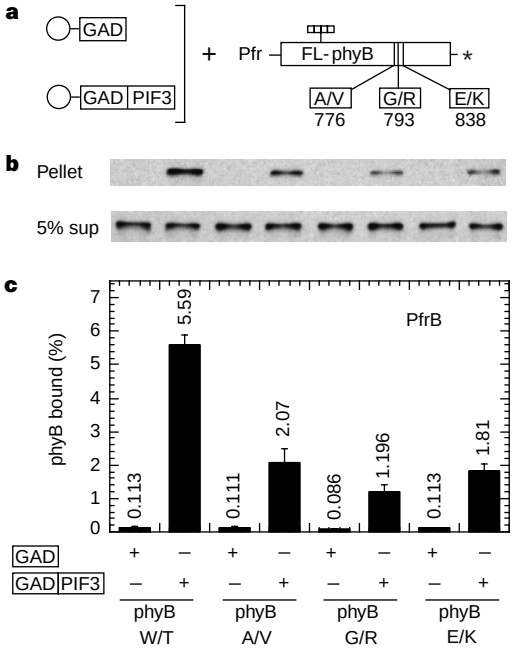

Figure 3 Missense mutations in phytochrome B impair PIF3 binding. **a**, Experimental design. Recombinant Gal4 activation domain (GAD) or GAD–PIF3 fusion protein was immobilized on beads (circles), then mixed with radioactively labelled (asterisk), chromophore-ligated (striped bar) wild-type full-length (FL) phyB in the Pfr form, or its mutant derivatives (A776/V, G793/R or E838/K) and incubated for 20 min at 4 °C in darkness before centrifugation. **b**, Autoradiography of pelleted phyB and 5% of supernatant fractions. **c**, Quantification of PIF3-bound, radioactive phyB shown in **b**, expressed as a per cent of total in supernatant and pellet. Each histogram corresponds to the sample above it in **b**, and represents the mean ± standard error of three experiments.

Methods

Construction of expression vectors. To produce bait proteins in *Escherichia coli*, the Gal4 activation domain (GAD) sequence was amplified by PCR using primers containing 5′ *Nde*I and 3′ *Sal*I restriction-enzyme cutting sites and cloned into the *Nde*I/*Xho*I sites of the pRSETB vector (Invitrogen). The expression vector construct for GAD–PIF3 was made as described[5]. To make T7-promoter driven *in vitro* transcription/translation templates, the full-length phytochrome B gene (*PHYB*) coding sequence was amplified by PCR and cloned into the pET-3c vector as described[16]. Full-length *PHYB* sequences bearing missense mutations were made by using a QuikChange site-directed mutagenesis kit with wild-type *PHYB* as template (Stratagene). The *PHYB* N-terminal sequence (corresponding amino acids 1–645) was amplified by PCR using primers containing 5′ *Nde*I and 3′ *Eco*RI restriction-enzyme cutting sites and cloned into *Nde*I/*Eco*RI sites of the pRSETC vector (Invitrogen). The *PHYB* C-terminal sequence corresponding to amino acids 645–1,211) was amplified by PCR and cloned into the pET-15b vector as described[5].

Expression of bait and prey proteins. Both GAD and GAD–PIF3 fusion proteins were expressed in *E. coli* host-strain BL21 cells. Overnight *E. coli* cultures were diluted 20-fold into fresh LB medium containing 50 μg ml^{-1} ampicillin, grown at 30 °C until they reached an absorbance at 600 nm of 0.6, and induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 h at 30 °C. Total protein was isolated by suspending the cells in 3 vol of PBS buffer (pH 7.4) containing 1 mM PMSF, 2 mM EDTA, 0.1% 2-mercaptoethanol, 5 mM benzamidine and 0.5% NP-40. The cell suspension was then sonicated for 4 × 20 s on ice with a 40-watt Tekmar sonic disruptor and cleared at 15,000*g* for 10 min at 4 °C. Total protein was analysed by SDS–PAGE and the expression of GAD or GAD–PIF3 was monitored and quantified by immunoblotting using a monoclonal antibody against the Gal4 activation domain (Santa Cruz Biotechnology). Full-length PHYB, and N- and C-terminal domains of PHYB were synthesized and labelled with ^{35}S-Met in the T'n'T *in vitro* transcription/translation system (Promega). The chromophore was attached to full-length or the N-terminal domain of PHYB by adding phycocyanobilin to the T'n'T reaction at 10 μM final concentration and incubating in darkness for 1 h at 4 °C. The phycocyanobilin was crude methanolysate, prepared as described[17].

Preparation of bait agarose beads. GAD and GAD–PIF3 were attached to agarose beads by precipitating 20 or 200 μg total protein of *E. coli* lysates containing similar levels of GAD and GAD–PIF3 using 1 μg monoclonal antibody against GAD and 20 μl protein-A agarose beads (Santa Cruz Biotechnology) incubated for 2 h at 4 °C in 0.3 ml PBS binding buffer (pH 7.4) as described[5]. The beads, with attached GAD or GAD–PIF3 proteins, were washed twice with PBS binding buffer without BSA and PMSF, and recovered by centrifugation at 3,000 r.p.m. for 5 min at 4 °C.

***In vitro* interaction assay.** Preparations of the prey moelcules containing full-length, N-, or C-terminal domains of PHYB synthesized in the T'n'T reaction were first precleared by adding 20 μl protein A/G plus agarose beads for 1 h in 0.3 ml PBS binding buffer. Precleared supernatants were then mixed with the bait agarose beads bearing equal amounts of GAD or GAD–PIF3. The immobilized bait proteins and bound phytochrome B proteins were present in roughly equimolar amounts in these assays. The mixtures were set on ice and received 5 min of red or far-red light illumination using an LED light source (QBEAM 2200) with a red light output of 74 μmol s^{-1} m^{-2} at 664 nm or a far-red light output of 84 μmol s^{-1} m^{-2} at 748 nm (Quantum Devices). The mixture was then incubated at 4 °C in darkness for different times, as specified in the figure legends. Agarose beads were then pelleted, washed four times with PBS binding buffer without BSA and PMSF, and the attached proteins were solubilized and analysed by 10% SDS–PAGE as described[5]. Five per cent of the supernatant from each pelleted sample was analysed simultaneously to estimate the per cent of total phytochrome bound. For quantification of radioactive protein, bands were excised and counted in a scintillation counter.

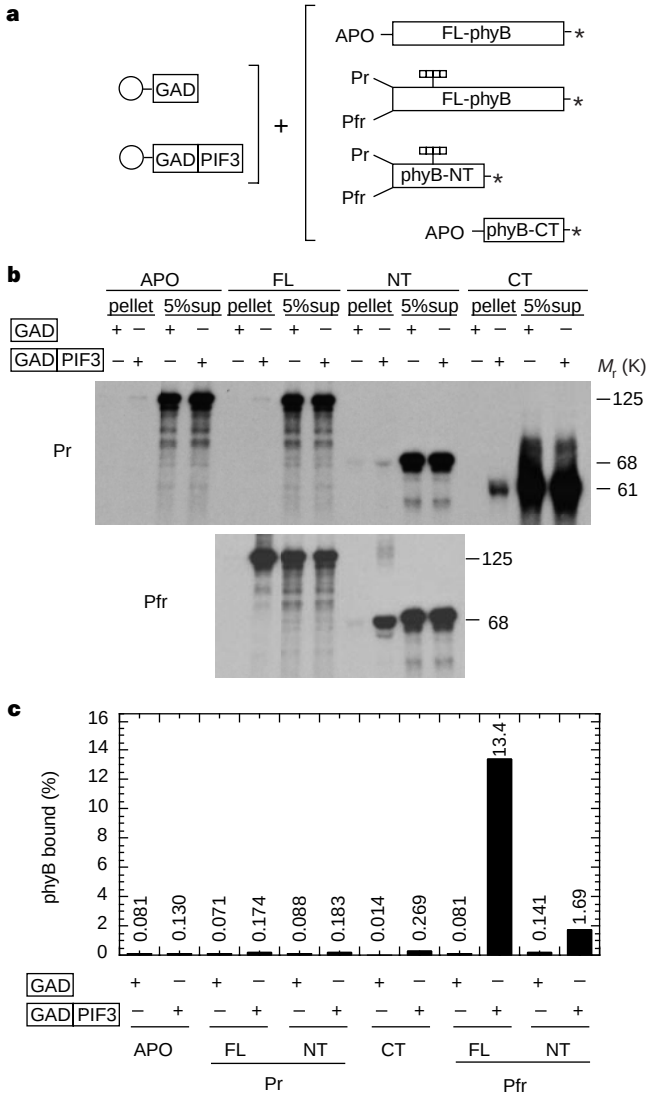

Figure 4 N- and C-terminal domains of phyB are both involved in PIF3 binding. **a**, Experimental design. Recombinant Gal4 activation-domain (GAD) or GAD–PIF3 protein was immobilized on beads, mixed individually with radioactive full-length (FL) or C-terminal-domain (CT) phyB apoproteins (APO), or with chromophore-ligated, FL or N-terminal-domain (NT) phyB in the Pr or Pfr forms, and incubated for 2 h at 4 °C in darkness before centrifugation. **b**, Autoradiography of FL phyB apoprotein, FL phyB, N-terminal and C-terminal domains of phyB in the entire pellet or 5% of supernatant (5% sup) following incubation as Pr or Pfr in the presence (+) or absence (–) of either GAD or GAD–PIF3. Exposure time for CT sample was 1.5 times longer than for the other samples. **c**, Quantification of PIF3-bound, radioactive phyB shown in **b**, expressed as a per cent of total in supernatant and pellet.

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Nucleosome mobilization catalysed by the yeast SWI/SNF complex

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The generation of a local chromatin topology conducive to transcription is a key step in gene regulation[1]. The yeast SWI/SNF complex is the founding member of a family of ATP-dependent remodelling activities capable of altering chromatin structure both *in vitro* and *in vivo*[2]. Despite its importance, the pathway by which the SWI/SNF complex disrupts chromatin structure is unknown. Here we use a model system to demonstrate that the yeast SWI/SNF complex can reposition nucleosomes in an ATP-dependent reaction that favours attachment of the histone octamer to an acceptor site on the same molecule of DNA (in *cis*). We show that SWI/SNF-mediated displacement of the histone octamer is effectively blocked by a barrier introduced into the DNA, suggesting that this redistribution involves sliding or tracking of nucleosomes along DNA, and that it is achieved by a catalytic mechanism. We conclude that SWI/SNF catalyses the redistribution of nucleosomes along DNA in *cis*, which may represent a general mechanism by which ATP-dependent chromatin remodelling occurs.

To investigate the possibility that the yeast SWI/SNF complex alters the position of nucleosomes on DNA, we generated a model template by ligating nucleosomal cores assembled onto radiolabelled 189-base-pair (bp) DNA fragments to one end of a 1,133-bp piece of naked DNA and separated the ligation products from unligated nucleosomes using streptavidin-coated magnetic beads (Fig. 1a). After washing the beads, barely any radiolabelled DNA was released in the absence of digestion by restriction enzyme (Fig. 1b, lane 1). Digestion of the template with *Nhe*I released the terminal 180 bp of the construct and electrophoresis on native gels revealed that most of the DNA released had the same mobility as nucleosomal DNA (Fig. 1b, lane 2). If the template was incubated with SWI/SNF complex in the presence of ATP before restriction-enzyme digestion (Fig. 1b, lane 6), most of the radiolabel was released as free DNA, indicating that the SWI/SNF complex promotes the removal of histones from this region of DNA. Consistent with previous studies[2], this ability of SWI/SNF to displace histones under these conditions was dependent on ATP (Fig. 1b, lane 5).

The SWI/SNF complex could remove histones from DNA by nucleosome disassembly, or by transferring histone octamers from one piece of DNA to another either by tracking along DNA or by a dissociative pathway[3]. To distinguish between these possibilities, we digested the template with restriction enzyme before incubating it with SWI/SNF complex. In this case, octamer removal was greatly reduced (Fig. 1b, lane 4). As these reactions in which the DNA was cut before incubation with SWI/SNF were otherwise identical to those performed on the intact template, SWI/SNF-mediated octamer displacement displayed a preference for the attachment of acceptor DNA in *cis*.

To determine the fate of nucleosomes displaced by the SWI/SNF complex, we generated a template containing restriction-enzyme digestion sites at positions that were successively more distant from the original position of the nucleosome (Fig. 2a). Figure 2b (lanes 2, 5, 8 and 11) shows that two distinct species were produced by digestion of this template: the mobility of the faster bands is consistent with release of the appropriate lengths of free DNA from the magnetic beads, the intensity and position of the slower band suggests that this piece of DNA was associated with a single histone octamer, which we confirmed after removing the histone octamer from its DNA by washing the beads with 2 M NaCl before restriction-enzyme digestion. This gave a single band with the same mobility as the faster band (Fig. 2b, lanes 1, 4, 7 and 10). Having established a way to monitor the position of histone octamer on the construct, we investigated the fate of nucleosomes after incubating them with SWI/SNF. We found that 45% of nucleosomes were displaced from the terminal 181 bp of template, but that when the template was digested with the restriction enzymes *Bgl*II, *Nco*I and *Eco*RI, each of which cuts further along the template, only 34, 18 and 4% of the histone octamers, respectively, were displaced (Fig. 2b, lanes 3, 6, 9 and 12). This ability of SWI/SNF to displace histone octamer from the end of the template but not from longer fragments indicates that it must have been redistributed away from the distal region and along the remainder of the fragment.

It has been inferred from changes in nucleosome positioning that nucleosomes move along DNA by sliding or tracking[4–9], but this movement could also result from dissociation of the octamer from

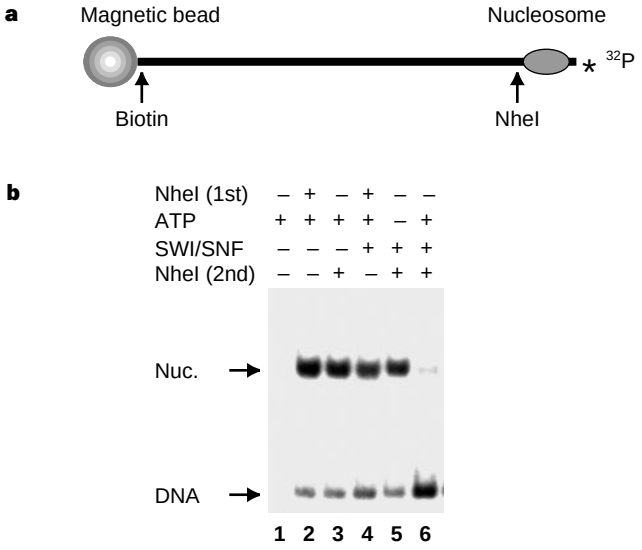

Figure 1 SWI/SNF displaces histones from DNA. **a**, Diagram of the substrate used in the sliding assay. **b**, The template does not enter the matrix of a polyacrylamide gel (lane 1) unless it has been digested with *Nhe*I, which releases the predominantly nucleosomal 3′ end (lane 2). If the template is incubated with SWI/SNF before cutting, most of the ^{32}P is released as free DNA (lane 6) in an ATP-dependent reaction (lane 5). In otherwise identical reactions, in which the substrate is cut before incubation with SWI/SNF, there is minimal histone displacement (lane 4). Nuc, *Nhe*I-digested nucleosomal DNA.